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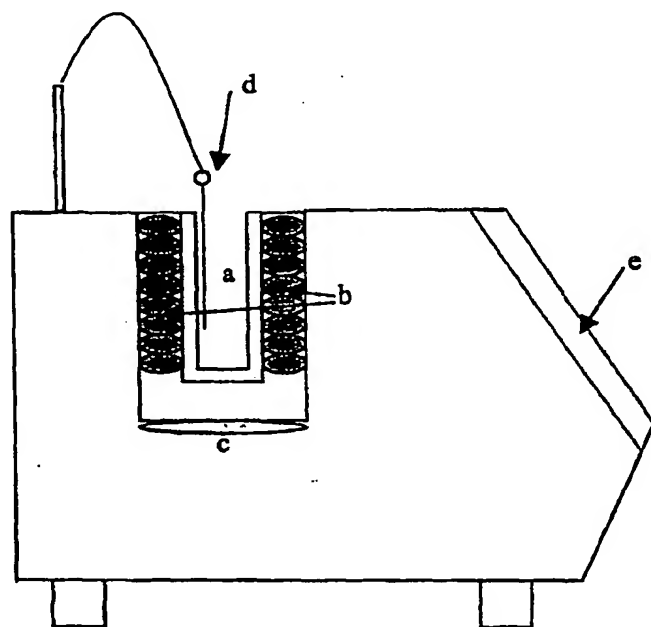
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[Continued on next page]

(54) Title: DEVICE FOR INTRODUCING PORES INTO BIOLOGICAL MATERIALS



(57) Abstract: The present invention relates to a device for creating pores in a biomaterial, such as a cell or tissue in a sample. The device uses an alternating magnetic field that increases the thermal or kinetic energy of magnetic particles present in the sample. The magnetic particles then create pores in the membrane surrounding the biostructure. Subsequently, the pores may be employed for the introduction of particles into the biostructure. In preferred embodiments the device is equipped with temperature control.

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DEVICE FOR INTRODUCING PORES INTO BIOLOGICAL MATERIALSField of the Invention

The present invention relates to a device for use in, inter alia, molecular biological work.

Background Art

5 In the fields of research biotechnology and biomedicine, it is often necessary to introduce a large molecule or a bioparticle into a biological structure, such as a bacterial cell. Cells and also viruses have an outer barrier for protection against the environment and
10 also a selective transport system for nutritive substances. In order to force natural protective mechanisms and introduce a substance which is not desirable for the target organism, some sort of chemical or physical treatment of the target cell is necessary. Examples of tech-
15 niques of forcing the outer cell membrane of cells, and where appropriate also the cell wall, are available in the fields of research genetic engineering and molecular biology.

When a new genetic code is transferred to a spe-
20 cially selected host cell, the technique is referred to as transformation or transfection. There is no general method to be used for all types of cells, but a technique is available for each cell type and purpose. Moreover it is not possible to transform all cell types using the
25 techniques that are currently available. In 1970, Mandel and Higa (*J. Mol. Bio.* 53: 159-162) reported that *E.coli* cells which had been pre-treated with CaCl_2 could be made to take up foreign DNA when subjected to a temperature shock. After that the method has been continuously de-
30 veloped, (see e.g. US patent application US97/01788). By exposing cells, during a fraction of a second, to an electric pulse of high voltage, pores in cell membranes open, referred to as electroporation (Zimmerman et al. *J. Membr. Biol.* 67: 165-82 (1983)), which is frequently

used as transformation technique. Bacteria, yeast and in some cases also mammalian cells and plant cells can, in specific conditions, be transformed by means of electroporation. Also in this case, a continuous development
5 of the technique is in progress (see patent applications US97/16721, US98/16042). In the two methods described above, the cell envelope is opened sufficiently long for the DNA molecule to enter the cell. The third and last developed method for transformation is so-called
10 lipofection (Old and Primrose, in *Principles of Gene Manipulation: An Introduction to Gene Manipulation*, Blackwell Science (1995)) where the foreign DNA is enclosed in/binds to a cationic liposome which fuses with the outer membrane of the target cell. There is one more
15 commercial technique for transformation of plant cells, where a plant part selected for the purpose is bombarded with small gold grains which are prepared with the foreign gene (Boynton J.E. et. Science 240, 1534-1538, 1988). Such gene transfer has been developed for trans-
20 formation of other tissues, such as bacteria, fungi, insect and mammalian cells (Johnston S.A. Nature 346, 776-777, 1990).

It is especially in the applications described above that the present invention can be used. However, it is
25 quite possible to use the inventive device to introduce other exogenic materials in applications, such as direct transfer of proteins, RNA molecules, fatty acids, peptides, medical preparations etc., to study the response of specific cells and viruses. Moreover the device ac-
30 cording to the invention is particularly suitable for lysis of cells for the purpose of carrying out lysis as well as identification and isolation of specific cellular components in one and the same method.

Summary of the Invention

35 Thus, the present invention relates to a device, characterised in that it comprises at least one coil in which a magnetic alternating field can be generated and

into which a sample can be inserted, where said magnetic field causes an increase of the thermal and/or kinetic energy of magnetically susceptible particles in said sample, the increased thermal and/or kinetic energy of said particles causing the formation of pores in biological membrane-enveloped structures which are to be found in said sample, said pores allowing introduction or extraction of bioparticles into/from said biological membrane-enveloped structures.

10 The method also relates to a method where a device according to the invention is used for specific lysis of cells. Furthermore the invention relates to a method where a device according to the invention is specifically used to modify the genetic code and/or metabolism of a host cell.

Brief Description of the Drawings

Fig. 1 is a principle sketch of the device according to the present invention.

20 Fig. 2 is an Example of an electronic current supply circuit.

Fig. 3 is an Example of the connection of a coil.

Fig. 4 shows an Example of a magnetically susceptible particle.

25 Fig. 5 shows a device which can generate a gradient field.

Detailed Description of the Invention

According to one aspect of the invention, the device is characterised in that said magnetic field has an alternating field direction of a frequency in the range 1-5 MHz.

According to another aspect, the device is characterised in that said magnetic field has a field strength of at least 1 mT.

35 According to one more aspect, the invention is characterised in that said magnetic field is non-homogeneous and has an alternating gradient field direction, the direction of said alternating gradient field being gene-

rated by two coils, and said sample is inserted between the coils.

According to one more aspect, the device according to the invention is characterised in that said coils are
5 supplied with alternating current of different frequencies.

According to yet another aspect, the device is characterised in that said coils are supplied with either the positive or the negative part of the supplied alternating
10 current.

According to another aspect, the device is characterised in that it is equipped with a thermostat for accurate temperature control of said coil or coils and/or said sample.

According to a further aspect, the device is characterised in that it is equipped with a variable timing for accurate control of the time during which said alternating current is on and during which said sample is
15 exposed to said applied magnetic field.

According to another aspect, the device is characterised in that it is equipped with a control system for accurate setting of strength and frequency of said alternating current.
20

The biological membrane-enveloped structures consist of, inter alia, body tissue, cells, bacteria, virus particles, organelles at a sub-cellular level, liposomes or proteins.
25

The bioparticles that are suitable for introduction into/extraction from membrane-enveloped structures are, inter alia, DNA molecules, RNA molecules, proteins, other biopolymers, peptides, chemical preparations, organic compounds, inorganic compounds or synthetic polymers or combinations thereof.
30

The technique on which the invention is partly based is a combination of magnetic nanotechnology and peptide chemistry. A magnetically susceptible particle having a size of between some ten micrometers and one nanometer is
35

used as a reagent in the technique. When such a particle is exposed to a certain magnetic field, it is made to vibrate and generate heat. Fig. 1 is a principle sketch of the present invention. The biological sample is mixed
5 with a reagent intended for the purpose and is then placed in a sample holder (a). The desired strength and frequency of the magnetic source (b) are adjusted, whereupon the desired temperature of the cooling element (c) is adjusted. The magnetic source is either one coil or
10 two coils, the sample being placed between them according to Fig. 5. The magnetic source and the sample holder are enclosed in an isolated unit, in which the temperature is determined by the cooling element. To ensure the correct temperature in the sample holder, a temperature sensor
15 (d) can be connected to the system. The variables temperature, strength and frequency of the magnetic field and treatment intervals are controlled and can be followed on a digital display (e).

Fig. 2 illustrates an example of an electronic
20 current supply circuit which comprises an oscillator (1) based on the circuit XR2206, whose output signal (2) is amplified by a power amplifying step (3), which is based on the circuit PBD 3548/1, whose output signal (4) can operate an alternating current (1MHz, 2A) through one or
25 more coils. An example of the connection of said coil is shown in Fig. 3, with an oscillating circuit consisting of a $2\ \Omega$ resistance (6), a 0.50 nF capacitor (7) and a 50 μ H coil (8), said circuit being supplied with alternating current (5). For a person skilled in the art it
30 is obvious that the above-described example illustrated in Figs 2 and 3 can easily be modified and that the same result can be obtained with the aid of alternative connections and coils.

Examples of magnetic materials that are used in the
35 method according to the invention are described in the patent literature, e.g. US 4,323,056 (Borelli et al). The magnetically susceptible particle and a possible configu-

ration are also illustrated in Fig. 4. The magnetically susceptible core (9) of the particle consists essentially of magnetite (iron oxide). Further the particle is coated with an outer layer (10) consisting of a derivatised polymer (Dextran), or a monolayer or alternatively bi-layer of derivatised fatty acids. The choice of the type (number of amino acid or carbohydrate units and sequence) of ligand 11 which is used for the derivatisation is individually adapted to each application of the magnetically susceptible particle, the effect of which can be still more amplified by its surface being further modified with one or more effector molecules 12.

By adding said particles to a cell suspension and then exposing the cell to a magnetic field with alternating field direction, instantaneous heating of the medium surrounding each magnetically susceptible particle is obtained. The heat induces a temperature shock in cell and cell membrane, which causes temporary openings in the cell membrane. The heat is induced quickly and homogeneously in the entire sample, which makes it possible for the sample and the cells to be exposed to treatment for a short while, which increases the survival frequency of the exposed cells. Example 1 describes transformation of *Escherichia coli*.

In a conventional transformation method involving a temperature shock, the test tube containing the cell suspension is exposed to a higher ambient temperature (42°C), whereby a temperature gradient arises from the test tube wall and into the sample, the composition of which requires a longer time than the method according to the present invention and which further implies that the cells that are located closest to the cell wall are exposed to the higher temperature for a longer time than those in the centre of the tube. Thus, some of the cells will die owing to the increased temperature while a fraction of the cells remain untreated. The method according to the invention circumvents this problem by the instan-

taneous heating round each particle in the sample holder. The effect is amplified if the particle is besides directed immediately to the cell envelopes via the ligand molecules on the surface of the particle. This is a great
5 advantage compared with conventional transformation methods where the balance between heat shock and cells death is important to the final result.

Furthermore, the field strength of the magnetic field can be varied in space, a so-called gradient field
10 which in combination with alternating field direction causes mechanical vibrations (kinetic energy increases) in the particles, which in combination with heat radiation (thermal energy) amplifies the effect of the particles on the cell membrane surrounding all cells (and
15 cell wall, where appropriate). The present invention describes a completely new method involving induction of heat or powerful introduction of shearing forces, or a combination thereof. The shearing forces initiate dislocations in the cell membrane owing to mechanical
20 fatigue, which results in breaks in cell membranes (and cell walls in the cases where the target cell is, for example, a bacterium). The method is based on the use of an alternating externally applied gradient magnetic field. A gradient field is provided with at least two
25 coils, which are supplied with either the positive or the negative part of the supplied alternating current, or alternatively said coils are supplied with alternating current of different frequencies. A device which can generate a gradient field is described in Fig. 5. The
30 functional principle is based on two coils (A) and (B) (with or without ferrite core) being arranged opposite to each other according to Fig. 5. A control unit (C) controls the current through the coils, so that the coils only one at a time have a current passing through their
35 windings. This current alternation, whose frequency is controlled by means of the oscillator (OSC), results in the coils alternately generating the gradient magnetic

fields (D) and (E) with different gradient directions. A magnetically susceptible particle (P) located between the coils will experience a gradient magnetic field with periodically alternating direction, which will induce a mechanical vibration. Alternatively, a gradient field can be generated when the two coils are supplied with current of two different frequencies. The difference in frequency between the current in the two coils controls the frequency of the alternation of the gradient.

By letting the magnetic treatment take place for a short while, conditions are created for a large number of surviving cells after treatment. As long as the cell envelope is open, the molecule which is to be transformed should be introduced into the cell. To optimise this procedure, the molecule can also be directed to the cell envelope. Both processes are effected by connecting recognition molecules for binding on the one hand to the cell surface and, on the other hand, to said molecule of one and the same ferromagnetic particle. Molecules, which on a biochemical basis can recognise and bind to biological structures of different kinds can be, for example, short synthetic peptides, parts of an antibody or an enzyme.

By connecting a recognition molecule of a target protein, such a recombinant protein, to the magnetically susceptible particles, the device according to the invention can be used for lysis and specific purification of said target protein in one and the same method. Compared with alternative techniques of lysis (mainly enzymatic and mechanical lysis), in combination with one or more purification steps, the use of the device according to the invention saves above all time, but also material.

The inventive device can advantageously be used on the one hand for a transformation method and, on the other hand, for purification of specific cell components, which makes the device unique. Regardless of the purpose, the method should take place while the coils are kept at

a constant temperature, which means that a cooling element and a temperature control should be incorporated into the controllable magnetic equipment. Moreover it is advantageous for the various potential fields of application of the device that the strength and frequency of the magnetic field as well as the time during which the sample is exposed to the treatment are variable.

EXAMPLE 1

The following Example describes a method for transformation of *Escherichia coli* (*E.coli*) with pUC18 plasmid:

100 μ l competent *E.coli* cells are mixed at 0°C with 500 μ g pUC18 dissolved in 30 μ l 0.05 M CaCl_2 . The sample is introduced into the sample container in the device according to the invention and incubated for 30 min at 0°C in the coil. Then the sample is treated for 30 s at 1MHz, 2A. 1 ml sterile LB broth is then added to the sample, which is then incubated in water bath for 1 h at 37°C. Subsequently the cells are spread on agar plates containing selection pressure, 50 μ g/ μ l ampicillin, for only the transformed bacteria to be obtained. The experiment should include a reference sample which does not contain pUC18 in order to assess survival and transformation frequency.

CLAIMS

1. A device, characterised in that it
5 comprises at least one coil, in which a magnetic alternating field can be generated and into which a sample can be inserted, where said magnetic field causes an increase of the thermal and/or kinetic energy of magnetically susceptible particles in said sample, the increased thermal
10 and/or kinetic energy of said particles causing the formation of pores in biological membrane-enveloped structures which are to be found in said sample, said pores allowing introduction or extraction of bioparticles into/from said biological membrane-enveloped structures.

15 2. A device as claimed in claim 1, characterised in that said magnetic field has an alternating field direction of a frequency in the range 1-5 MHz.

3. A device as claimed in claim 1 or 2, characterised
20 acterised in that said magnetic field has a field strength of at least 1 mT.

4. A device as claimed in any one of claims 1-3, characterised in that said magnetic field is non-homogeneous and has an alternating gradient field direction, the direction of said alternating gradient field
25 being generated by two coils, and said sample is inserted between the coils.

5. A device as claimed in claim 4, characterised
30 t e r i s e d in that said coils are supplied with alternating current of different frequencies.

6. A device as claimed in claim 4, characterised
t e r i s e d in that said coils are supplied with either the positive or the negative part of the supplied alternating current.

7. A device as claimed in any one of claims 1-6, characterised in that it is equipped with a thermostat for accurate temperature control of said coil or coils and/or said sample.

5 8. A device as claimed in any one of claims 1-7, characterised in that it is equipped with a variable timing for accurate control of the time during which said alternating current is on and during which said sample is exposed to said applied magnetic field.

10 9. A device as claimed in any one of claims 1-8, characterised in that it is equipped with a control system for accurate setting of strength and frequency of said alternating current.

15 10. A device as claimed in any one of claims 1-9, characterised in that said biological membrane-enveloped structures consist of body tissue, cells, bacteria, virus particles, organelles at a sub-cellular level, liposomes or proteins.

20 11. A device as claimed in any one of claims 1-10, characterised in that said bioparticles are DNA molecules, RNA molecules, proteins, other biopolymers, peptides, chemical preparations, organic compounds, inorganic compounds or synthetic polymers or combinations thereof.

25 12. A method in which the device as claimed in any one of claims 1-10 is used for specific lysis of cells.

13. A method in which the device as claimed in any one of claims 1-10 is specifically used to modify the genetic code of a host cell and/or metabolism.

FIG. 1.

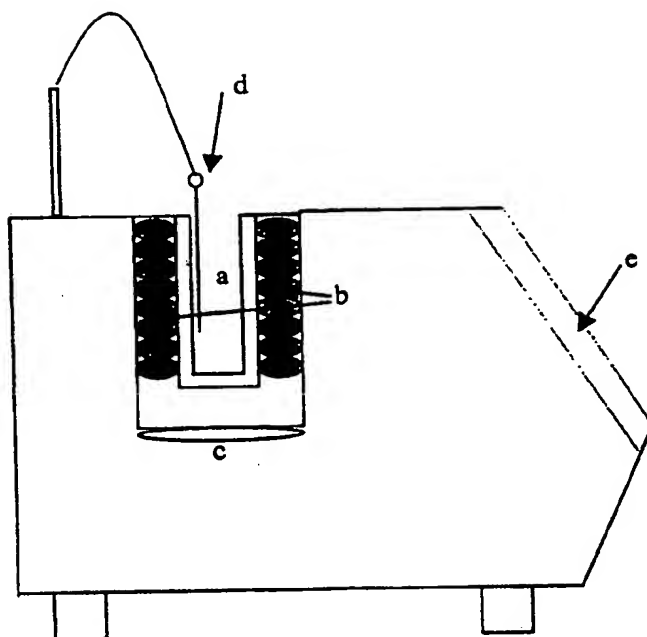


FIG. 2

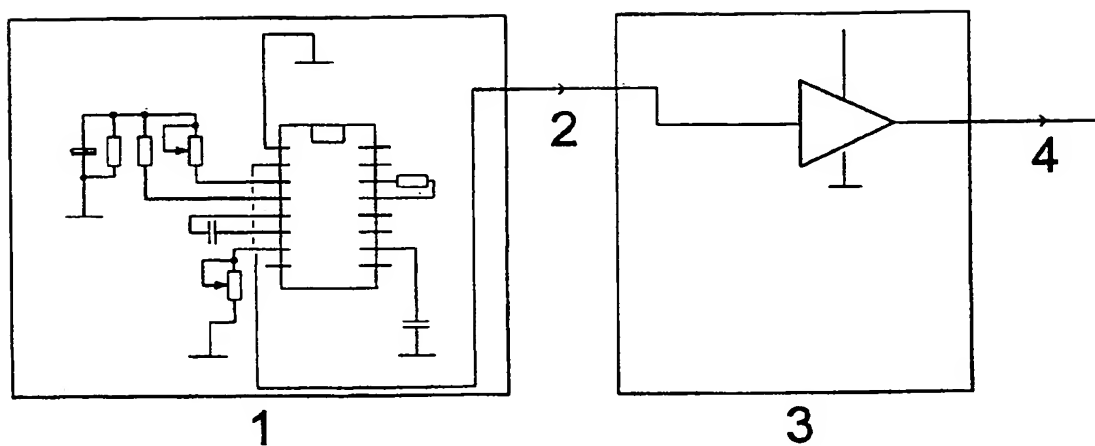


FIG. 3

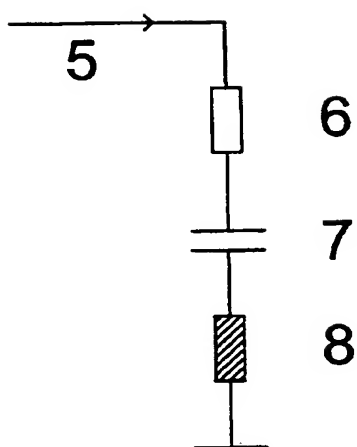


FIG. 4.

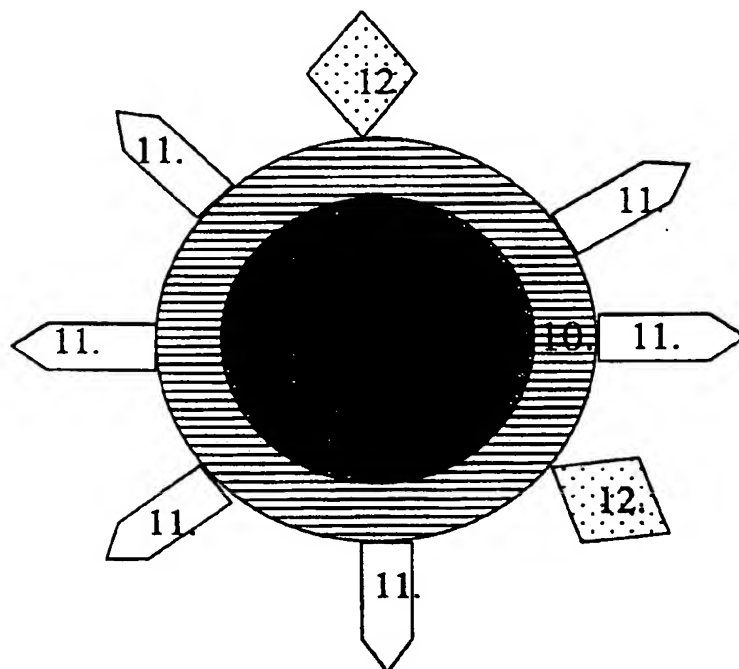
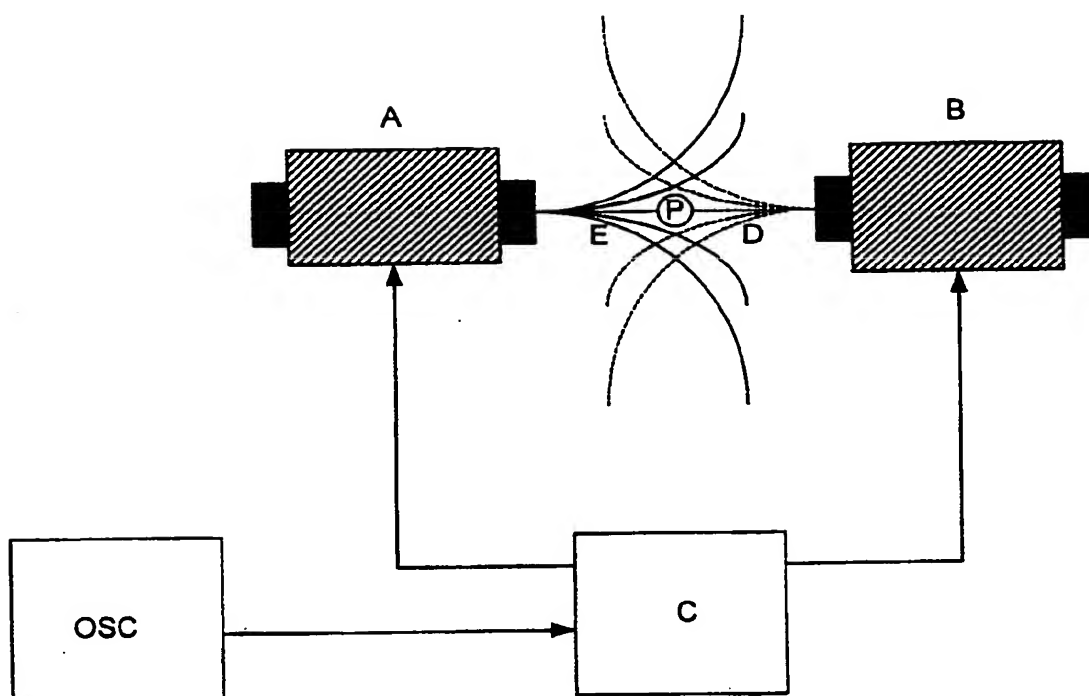


FIG. 5.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01743

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12M 1/42, C12N 13/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12M, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biophysical Journal, Volume 71, August 1996, Timothy E. Vaughan et al, "Energetic Constraints on the Creation of Cell Membrane Pores by Magnetic Particles" page 616 - page 622 --	1-13
X	Bioclectrochemistry and Bioenergetics, Volume 46, 1998, Timothy E. Vaughan et al, "Molecular change due to biomagnetic stimulation and transient magnetic fields: mechanical interference constraints on possible effects by cell membrane pore creation via magnetic particles" page 121 - page 128 -- -----	1-13

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier application or patent but published on or after the international filing date
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01743

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **1-11**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see next sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/01743

Present claims 1-11 relate to an apparatus defined by reference to a desirable characteristic or property, achieved by generating an alternating magnetic field in the apparatus. Thereby, pores in biological structures covered by membranes are created by magnetic particles present in a sample. The claims cover all apparatus having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and / or disclosure within the meaning of Article 5 PCT for only a very limited number of such apparatus. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lacks clarity (Article 6 PCT). An attempt is made to define the apparatus by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out based on the disclosures of the description.

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